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OXYGEN POISONING IN THE ANNELID *TUBIFEX TUBIFEX* I. RESPONSE TO OXYGEN EXPOSURE [1]

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The freshwater Annelid *Tubifex tubifex* lives partially submerged in the bottom mud of ponds and slow-moving streams where the concentration of dissolved oxygen may become very low. During periods when oxygen is limited, these worms extend their tails far out of the mud and move with vigorous stirring motions to provide a more oxygen-rich environment (Alsterberg, 1922).

T. tubifex has no specialized respiratory system and is dependent on diffusion through the body surface for its oxygen supply. While *T. tubifex* is able to live in environments with extremely low oxygen tensions, it is also capable of maintaining a constant oxygen consumption when the oxygen tension is increased above atmospheric levels even to concentrations as great as one atmosphere oxygen pressure (Harnisch, 1935; Koenen, 1951; Walker, 1955). Anderson (1956) found that exposure to one atmosphere oxygen pressure for ten days is lethal to *T. tubifex* and also inhibits regeneration; 10^{-5} *M* cyanide stimulates some phases of regeneration. When regenerating worms are exposed to one atmosphere oxygen pressure in the presence of cyanide, survival is enhanced and the inhibition of regeneration by oxygen is partially reversed (Anderson, 1956).

The blood of *T. tubifex* contains hemoglobin in solution which has an extremely high affinity for oxygen (Fox, 1945). The role of hemoglobin in this organism is a matter for conjecture. Manwell (1959) has suggested that it may function to prevent oxygen poisoning in organisms such as *T. tubifex* which normally function at very low internal partial pressures of oxygen by protecting against development of high internal oxygen concentrations. Palmer (1968) on the other hand, has evidence that hemoglobin does function in oxygen transport but only when the external oxygen concentration falls below the critical tension.

In recent years reports have appeared of the effects of high oxygen pressure on subcellular organelles and upon electron transport systems both *in vitro* (Chance, Jamieson and Coles, 1965; Chance, Jamieson and Williamson, 1966; Jamieson and Chance, 1966) and *in vivo* (Chance *et al.*, 1965; Chance *et al.*, 1966). Whether the short-term reversible changes observed in these systems are related to the longer-term irreversible effects of oxygen in the whole organism is not readily apparent. It was therefore of interest to examine some of the factors influencing the pattern of oxygen poisoning in a whole organism which would be readily amenable to environmental manipulations. Because *T. tubifex* has considerable

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capacity for altering its metabolic patterns and responses in various environmental and physiological conditions, it appeared to be an interesting organism to use for such studies.

Materials and Methods

Worms were collected from a local drainage ditch and were stored covered with water in the stream bottom mud in a pail in the laboratory. The water used in this study was tap water which had been passed through activated charcoal filters. During initial experiments some difficulties were encountered in maintaining the worms for long periods of time. Contamination of the tap water with metal ions such as copper known to be toxic to *T. tubifex* (Jones, 1938) was suspected, therefore 0.05 g of the metal chelator disodium dihydrogen ethylenediamine tetraacetic acid (Hach Chemical Co.) per liter was added to all water used in these experiments. Immediately before use the worms were separated from the mud and each worm was anesthetized in 0.2% chloretone and examined under a compound microscope for species, absence of injury, lack of external parasites and state of regeneration. Two species of Oligochaeta were usually found together in the same collection. *T. tubifex* was identified during the microscopic examination by the setae pattern; four to five prominent capilliform setae together with usually six pectinate setae in the dorsal bundles of segments anterior to the clitellum and bifurcate setae only in the ventral bundles. The other species had short, bifurcate setae only in both the dorsal and ventral bundles. These organisms were identified as belonging to the genus *Limnodrilus*. Both normal and regenerating worms were included in the initial experimental series. All worms used in later experiments were cut for regeneration during the examination period in chloretone in order to ensure a more uniform experimental population. Each worm was placed into five ml water in an individual depression drilled in a block of household-type USP paraffin (Parawax, Standard Oil Co.); each worm was therefore isolated during the entire experimental procedure. This technique expedited the scoring of post-exposure responses to oxygen and eliminated the possibility of effects of contamination from other injured worms.

The chamber used for oxygen exposure was built of steel pipe and fitted with gas inlet and outlet valves and a pressure gauge. The chamber was flushed with oxygen (Linde, USP) for five minutes prior to increasing the pressure to the exposure conditions of four atmospheres absolute. When the effect of pressure alone was tested, the chamber was not flushed and nitrogen (Linde, high purity, dry) was added to the atmosphere of air already present until four atmospheres absolute were attained. All exposures were made at four atmospheres absolute; the dose was varied by varying the time of gas exposure. No difference in worm survival could be correlated with short or long periods of decompression, but after the initial experimental series a standard decompression of two pounds per minute was used.

Worms were exposed to oxygen in the paraffin blocks. After gas exposure the blocks were stored on the laboratory table under loosely fitting bell jars to prevent contamination and retard evaporation. Worms were examined several times daily for the first two or three days following oxygen exposure; afterwards,

they were examined approximately once daily until at least 12 days had elapsed. The time intervals presented in the tables were chosen to correspond as closely as possible to times at which the actual observations were made. When observations were not made at these particular times, the data were obtained by graphic interpolation from the actual survival data immediately before and after the time given.

When the worms were exposed to interrupted oxygen doses, they were returned to atmospheric conditions during the interruption. The duration of interruption was taken as the time from the end of the first decompression until pressure was attained for the second oxygen exposure. Post-exposure survival was scored at various intervals measured from the end of the second decompression.

Results

1. *Response of* T. tubifex *to four atmospheres absolute oxygen pressure*

A. *Gross appearance of oxygen-treated worms.* Worm behavor on removal from the oxygen chamber was closely related to the duration of oxygen exposure. The first noticeable response to oxygen was marked hyperactivity (one-hour exposure). The worms were extended and moving with vigorous stirring motions. Worm hyperactivity changed to a slower occasional whip-like thrashing for oxygen exposures of two to four hours. Following five- and six-hour exposures the worms were moving slowly and some were in a partially coiled or contracted state. After eight hours of oxygen treatment all worms contracted showing little movement. Any occasional movements were tortuous. After nine hours the first damage was apparent. Following longer oxygen exposures, worms were coiled, showed little movement, appeared swollen segmentally and occasionally had lost posterior segments. Changes from these initial responses occurred only after several hours when the worms either showed definite signs of recovery or began to disintegrate. As damage progressed the worms became swollen segmentally and appeared transparent with prominent and enlarged blood vessels. When the worms disintegrated they ruptured usually near the intersegmental septum, and cells, blood and body fluids were extruded. Any remaining tissue was a grey, opaque, motionless mass. In many cases complete dissolution occurred.

B. *Time course of mortality following oxygen exposure.* Worm survival data fell into one of three distinct patterns. Oxygen exposures of from one to eight hours resulted in negligible mortality throughout the two-week observation period. Mortality of these groups was indistinguishable from unexposed controls. Approximately half the worms died following exposures of nine to 12 hours; of these deaths virtually none occurred during the first six hours but approximately half occurred during the first 24 hours after exposure. Exposure times of 13 hours or more proved lethal to almost all worms during the first 120 hours. After these high doses approximately half the deaths occurred during the first six hours but many deaths were delayed beyond 24 hours. Representative survival curves are presented in Figure 1. Since a significant proportion of the deaths occur more than 48 hours after exposure, it is clear that observation times of greater than 48 hours must be used in the evaluation of factors influencing mortality of worms exposed to hyperbaric oxygen. When the data for all the worms within each of the high, intermediate and low survival groups and for the unexposed controls were pooled and

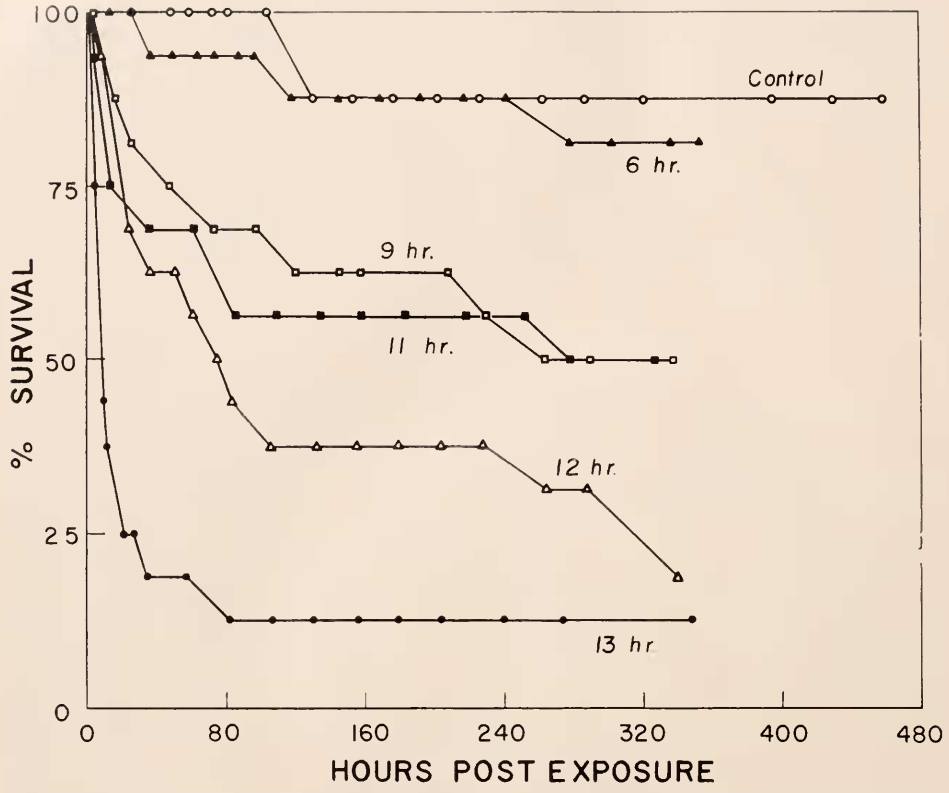

FIGURE 1. Post-exposure survival patterns of *T. tubifex* following low (1 to 8 hour), intermediate (9 to 12 hour) and high (13 to 15 hour) oxygen doses and survival pattern of unexposed controls.

hourly mortality rates calculated, high mortality rates within the first six to eight hours postexposure were found for both high and intermediate oxygen doses. In both the high dose group and the intermediate dose group a second peak of mortality occurred after a few days.

2. *Effect of pressure without increased oxygen*

Two experiments were run to distinguish the effects of pressure from those specifically related to oxygen. Two groups of worms cut for regeneration (24 worms per group) were exposed to three atmospheres of nitrogen added to one atmosphere of air. One group was exposed for 18 hours and one for 30 hours. Eight unexposed worms served as controls for each experiment. Worms removed from nitrogen were indistinguishable from unexposed controls after nitrogen treatment for as long as 30 hours. They showed neither the hyperactivity nor the contraction with lack of movement typical of oxygen-treated worms. Mortality of nitrogen-exposed worms after as long as 33 days was no greater than of unexposed controls. Results are presented in Table 1.

3. *Interrupted exposure to hyperbaric oxygen*

The effect on survival of interrupting the oxygen exposure for various time periods was determined. A 16-hour exposure was chosen since this dose usually resulted in complete worm mortality but an exposure of half this duration was sublethal. Oxygen exposures of one half the lethal dose separated by various intervals could therefore be used to indicate the occurrence of repair processes. Worm groups were exposed to oxygen for eight hours, returned to atmospheric conditions and re-exposed to oxygen for a second eight-hour period.

The relationship between survival at 120 hours after oxygen exposure and the duration of interruption is presented in Figure 2. Interruption of the oxygen exposure clearly leads to an increase in worm survival indicating repair of some type of early damage. This repair process is one half maximal in four hours and maximal in approximately 16 hours. The time course of mortality and the survival at

TABLE I

Survival of T. tubifex *following exposure to three atmospheres nitrogen plus one atmosphere air (24 worms per nitrogen-exposed group)*

Duration of nitrogen exposure (hours)	Duration of experiment (hours)	Post-exposure survival (per cent)				
		24 hours	48 hours	168 hours	200 hours	Final
Experiment 1						
0	343	100.0	100.0	100.0	87.5	87.5
18	343	100.0	100.0	87.5	87.0*	83.3
Experiment 2						
0	792	100.0	100.0	82.0*	62.5	50.0
30	792	95.9	95.9	75.0*	62.0*	41.6

* By interpolation.

120 hours for two half-lethal oxygen doses interrupted by four, six, eight, 12 and 20 hours closely resemble those resulting from single oxygen doses of from nine to 12 hours. A 16-hour interruption resulted in 100% survival.

4. *Adaptation*

In view of the ability of *T. tubifex* to withstand alterations in environmental conditions, it seemed possible that these worms might adapt to conditions of increased oxygen tensions in their environment. This possibility was tested in the following manner. Worms were separated from the stock bucket, cut for regeneration and placed into five ml water in the paraffin blocks on the laboratory table under aluminum foil for 14 days. These worms were therefore more directly exposed to atmospheric oxygen concentrations without the protective mud environment available to them in the stock bucket or in nature. Before oxygen exposure these worms were recut for regeneration and exposed to an 18-hour oxygen dose along with worms freshly separated from the stock bucket and cut for regeneration. Results of two experiments are presented in Table II. Worms exposed to increased oxygen tensions in shallow depressions develop a significant resistance to

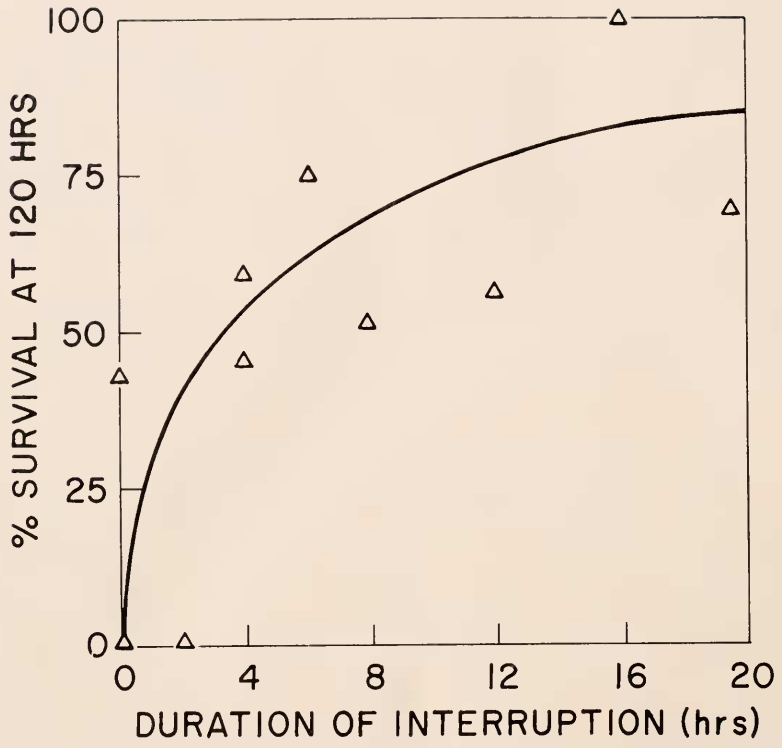

FIGURE 2. Per cent survival at 120 hours post exposure of *T. tubifex* following administration of two eight-hour oxygen doses interrupted by an interval in air.

hyperbaric oxygen while those exposed to oxygen immediately after separation from the stock bucket were markedly oxygen sensitive. This increased resistance to oxygen might represent true adaptation of the worms to conditions of higher oxygen tension or be the result of a conditioning of the environmental medium. To test these alternatives an exchange experiment was run. After 17 days of

TABLE II

Survival after 18-hour oxygen exposure of freshly-separated and of laboratory-adapted T. tubifex (*16 worms per group*)

Worm group	Post-exposure survival (per cent)			
	20 ± 1 hours	42 ± 1 hours	92 ± 3 hours	196 ± 12 hours
Experiment 1				
Freshly-separated	6.3	6.3	6.3	0.0
Adapted	100.0	100.0	93.8	93.8
Experiment 2				
Freshly-separated	43.8	31.3	6.3	6.3
Adapted	100.0	100.0	87.5	87.5

storage in paraffin blocks in the laboratory, worms were cut for regeneration. Some of these worms were replaced in the same depressions. Others were transferred into depressions containing fresh water. Freshly-separated and cut worms were placed either into fresh water or into the water from which the laboratory-stored worms had been removed. These four worm groups were simultaneously exposed to oxygen for 20 hours. Results are given in Table III.

As in the previous experiments, the worms kept in shallow depressions in the laboratory were markedly oxygen resistant. Transfer to fresh water may slightly decrease this resistance. There is no significant protection of freshly-separated worms by the conditioned water.

TABLE III

Survival of T. tubifex *after 20-hour oxygen exposure as affected by adaptation of worms and conditioning of medium (11 to 22 worms per group)*

Worm group	Post-exposure survival (per cent)			
	21 hours	47 hours	138 hours	229 hours
Freshly-separated, fresh water	0.0	0.0	0.0	0.0
Freshly-separated, conditioned water	9.0	9.0	—*	—*
Adapted, fresh water	68.2	68.2	59.1	59.1
Adapted, conditioned water	94.7	94.7	94.7	84.3

* No additional data.

DISCUSSION

The results presented here indicate that increasing duration of exposure to hyperbaric oxygen leads to both increased total mortality and increased rate of mortality of *T. tubifex*. Most published work on oxygen exposure of whole organisms has emphasized the immediate lethal effect of oxygen treatment. Immediate mortality after hyperbaric oxygen exposure has been reported for parasitic protozoa (Cleveland, 1925; Cleveland and Burke, 1956), *Paramecia* (Wittner, 1957; Gerschman, Gilbert and Frost, 1958a), *Drosophila* (Williams and Beecher, 1944), mice (Gerschman, Gilbert and Caccamise, 1958b), rats (Ozorio de Almeida, 1934; Stadie, Riggs and Haugaard, 1945), rabbits (Hederer and Andre, 1940) and dogs (Paine, Lynn and Keys, 1941). The present work demonstrates that the lethal effect of hyperbaric oxygen may not become manifest for several hours and that some lethally-exposed animals may survive for many days. Any proposed mechanism for oxygen poisoning may require a sequence of events before the final changes can be recognized.

Analysis of mortality rates indicates that two peaks of mortality occur following exposure to hyperbaric oxygen. This suggests that there may be two modes of death of *T. tubifex* following oxygen exposure or that within the worm population there are two types of individuals with different sensitivities to hyperbaric oxygen.

Since nitrogen at pressures equivalent to that of hyperbaric oxygen produced no significant mortality, the effects reported here can be attributed to oxygen and not to pressure itself.

Interruption midway through a 16-hour total exposure markedly increased the survival of *T. tubifex*. Interrupting oxygen exposures with an interval in air has also been reported to benefit dogs (Paine *et al.*, 1941), rats (Barach, Eckman, Oppenheimer, Rumsey and Soroka, 1944; Ackerman and Brinkley, 1966), guinea pigs (Penrod, 1956), mice (Wright, Weiss, Hiatt and Rustagi, 1966), rabbits (Ackerman and Brinkley, 1966) and *Drosophila* (Fenn, Henning and Philpott, 1967a; Fenn, Philpott, Meehan and Henning, 1967b). Recovery from eight-hour, half-lethal oxygen doses in *T. tubifex* is rapid being half maximal in four hours and maximal in approximately 16 hours. Since a 16-hour interruption led to 100% survival but a 20-hour interruption permitted only a 70% survival, it is not clear whether recovery goes to completion. Repair processes may be only partially complete. These findings appear to be similar to those reported by Fenn *et al.* (1967b) for *Drosophila*. It is possible that the kinetics of repair may be different for recovery from sublethal oxygen doses than for processes having the maximum effect. Incomplete recovery may be considered to be a residual injury. The interpretation is complicated by the occurrence of adaptation in this organism.

Adaptation to increased oxygen tensions has been reported previously. An increased tolerance to hyperbaric oxygen following an initial oxygen exposure (Smith, Heim, Thompson and Drinker, 1932) or after a gradual increase in the oxygen concentration extending over a number of days (Barach *et al.*, 1944) has been observed in the rat. The present report appears to be the first demonstration of adaptation to hyperbaric oxygen in an invertebrate.

Some invertebrates, *Daphnia* (Fox, 1955), *Chironomus* larvae (Fox, 1955) and *Artemia* (Gilchrist, 1954) respond to lowered environmental oxygen tensions with an increased synthesis of hemoglobin. The fact that *T. tubifex* does not synthesize additional hemoglobin under these conditions (Fox, 1955) lends support to Manwell's (1959) suggestion that hemoglobin in this organism may function as a buffer to protect against development of high oxygen concentrations in the tissues. It would be of interest to test for a correlation between the environmental oxygen concentration and the level of hemoglobin present in this organism.

I wish to express my deepest appreciation to Dr. Howard S. Ducoff for his advice and guidance throughout this study, to Dr. John D. Anderson for his continued interest and to the Department of Physiology and Biophysics for the use of their facilities.

Summary

1. Exposure to hyperbaric oxygen caused *T. tubifex* to become hyperactive. After high doses of oxygen the worms became highly coiled and contracted and showed little movement. The behavioral response to oxygen exposure became complete only after several hours when the animals either disintegrated or began to resume normal behavior.

2. Exposure to four atmospheres absolute oxygen pressure for 13 to 15 hours caused high mortality of *T. tubifex*. Only an occasional worm survived. Exposure to four atmospheres absolute oxygen pressure for nine to 12 hours killed approximately half of the treated animals. Exposure to four atmospheres absolute

oxygen pressure for eight hours or less caused no greater mortality than in unexposed controls.

3. The lethal effects of oxygen exposure developed only after several hours. The lethal effect of a nine- to 12-hour exposure was half maximal in 24 hours and the effect of a 13- to 15-hour exposure in six hours. Some lethally-exposed worms survived for as long as 120 hours after 13- to 15-hour oxygen exposures.

4. Exposure to three atmospheres nitrogen added to one atmosphere of air for as long as 30 hours resulted in no more deaths than in unexposed controls and caused no change in worm behavior.

5. Interruption of a 16-hour oxygen exposure with return to atmospheric conditions enhanced survival of oxygen-treated *T. tubifex*. Increased duration of interruption led to increased survival of oxygen-treated worms. Recovery was half maximal in four hours and maximal in approximately 16 hours.

6. Adaptation to hyperbaric oxygen occurs in *T. tubifex* following several weeks of exposure to atmospheric oxygen tensions in shallow containers without a protective mud environment.

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